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Structural characterization and macrophage activation of a hetero-galactan isolated from *Flammulina velutipes*

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ABSTRACT

We isolated and purified a new polysaccharide (WFVP-N-b1) with a molecular weight of 20 kDa from *Flammulina velutipes*. Results showed that WFVP-N-b1 is composed of an α (1 → 6)-linked D-galactan backbone and branched at the O-2 of its Gal*p* residues by an α-D-(1 → 6)-linked Man*p* attached to t-β-D-Glc*p* or t-α-D-Fuc*p* side chains. WFVP-N-b1 can significantly induce cytokines secretion and release of toxic molecules. On a cellular level, WFVP-N-b1 is recognized by Toll-like receptor 4 (TLR4). Thereby, the hetero-galactan increased the phosphorylation of mitogen-activated protein kinases (MAPKs) and Akt, promoted degradation of IκB-α and the nuclear translocation of the NF-κB p65 subunit. Importantly, our results indicate that WFVP-N-b1 activated macrophage is mediated by autophagy, as blockade of WFVP-N-b1-induced autophagy by Baf-A1 significantly decreases macrophage activation. This is the first report that hetero-galactan-induced macrophage activation is mediated by autophagy. Collectively, WFVP-N-b1 activated RAW264.7 cells through MAPKs, autophagy, and Akt/NF-κB signaling pathways via TLR4 receptor.

1. Introduction

Macrophages have a unique niche in the immune system by initiating innate immune responses. Following activation, macrophages can neutralize foreign substances, infectious microbes and cancer cells directly through phagocytosis and indirectly by secreting pro-inflammatory cytokines and cytotoxic molecules (Kim et al., 2012). In addition, macrophages also exert an important role as an interface between innate and adaptive immunity (Varin & Gordon, 2009). Thus, macrophage activation could present itself as a hopeful strategy to improve host immunity. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), Dectin-1 and complement receptor type 3 (CR3), are required for macrophages to store cognate, extracellular stimulators (Gordon, 2002).This recognition triggers intracellular signaling cascades including PI3K/Akt, NF-κB, mTOR, MAPK pathways (Fang et al., 2017; Wang et al., 2016; Wei et al., 2016), and thereby prompts macrophages to secrete downstream effector molecules such as ROS, NO, TNF-α, IL-6 and IL-1β etc. (Deng et al., 2016; M.M. Liu, Zeng, Li, & Shi, 2016), thus strengthening host immunity.

Recently, autophagy was found to be involved in immunity, it can act as a direct effector by eliminating invading pathogens, regulating innate pathogen recognition, contributing to controlling B- and T cell development (Zhong, Sanchez-Lopez, & Karin, 2016). It also can modulate inflammasome activation (Zhang, Qi, Guo, Zhou, & Zhang, 2016) and cytokines secretion of TNF-α, IL-6 and IL-1β (Harris et al., 2011). Growing evidence suggests that polysaccharides and oligosaccharides from natural sources have potential as immunomodulators by recognizing macrophage cell surface receptors and initiating signal transduction (Chen & Seviour, 2007; Zhang, Liu, Peng, Han, & Yang, 2014). However, limited studies have shown that polysaccharide can induce autophagy in macrophages (Chechushkov et al., 2016; Ohman et al., 2014).

Mushrooms contain many bioactive compounds which make them potential sources for pharmaceutical drug discovery and functional food. *Flammulina velutipes* (*F. velutipes*), also known as golden needle mushroom or enokitake, is the fourth most popular edible mushrooms in the world (Jing et al., 2014). In recent studies, *F. velutipes* has been shown to be a low calorie mushroom with high levels of essential amino acids, vitamins, fiber, and polysaccharides (Leifa, Pandey, & Soccol, 2000). Due to its beneficial bioactivities, *F. velutipes* has been widely used as a food additive, cosmetic ingredient and pharmaceutical material (Tang et al., 2016).

Abbreviations: TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; MAPKs, mitogen-activated protein kinases; ERKs, extracellular signal-regulated kinases; JNK, c-Jun NH2-terminal kinase; DCFH-DA, dichlorofluorescein diacetate; PI, propidium iodide; ROS, reactive oxygen species; Baf-A1, bafilomycin A1; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitricoxide

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Polysaccharides have attracted increasing attention for nutrition and food science because of their substantial medicinal properties and non-toxic side effects (Wasser, 2017). Polysaccharides derived from *F. velutipes* have been reported to be effective antioxidants (Y. Liu et al., 2016), anti-tumor drugs (Leung, Fung, & Choy, 1997), and T lymphocyte proliferation promoting agents (Yan, Liu, Mao, Li, & Li, 2014). It has been reported that the impure polysaccharides from *F. velutipes* could stimulate macrophage cell proliferation and phagocytosis (Shi, Yang, Guan, Zhang, & Zhang, 2012). Overall, these findings suggest that *F. velutipes*–derived polysaccharides can help to regulate the immune system. However, most studies of polysaccharides from *F. velutipes* have focused on identification of homo-glucans, such as β-1 → 3-glucan (Smiderle et al., 2006), α-1 → 4-glucan (Pang et al., 2007; Yin et al., 2010). Few studies about hetero-galactans from *F. velutipes* have been reported (Smiderle, Carbonero, Sassaki, Gorin, & Iacomini, 2008; Zhang, Xiao, Deng, He, & Sun, 2012).

Recently, we isolated and purified a novel hetero-galactans (WFVP-N-b1) from *F. velutipes*. In addition, there is limited knowledge as to the chemical/structural characterization and immunomodulatory activity in macrophages. Therefore, the present study is aimed at providing this information and identifying the molecular mechanism responsible for macrophage activation.

2. Materials and methods

2.1. Materials

Fruiting bodies of *F. velutipes* were collected at the Changbai mountain area in Jilin Province, PR China and were identified by using rDNA-ITS sequencing analysis. Sepharose CL-6B and Sephadex G-100 were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). LPS was obtained from Sigma (Sigma Aldrich, St Louis, MO, USA). Antibodies against Dectin-1, TLR2 and TLR4 were acquired from R&D Systems, Inc. (Minneapolis, MN, USA), Abcam (Cambridge, MA, USA) and BD (San Jose, CA, USA), respectively. Other Antibodies were purchased from Cell Signaling (Danvers, MA, USA). ToxinEraser™ Chromogenic LAL Endotoxin Detection Assay Kit was purchased from GenScript (Nanjing, China). ELISA kits were obtained from Boster Biological Technology (Wuhan, China). BAY11-7082, SP600125, U0126 and SB203580were acquired from Selleck (Shanghai, China). TAK-242 was acquired from MCE (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of WFVP-N-b1

Fruiting bodies were extracted using distilled water at 100 °C for 4 h. Supernatants were concentrated to small volumes under vacuum at 60 °C, followed by addition of 95% ethanol to a final concentration of 75% in order to precipitate polysaccharides. These were then collected by centrifugation (4000 rpm, 15 min) and vacuum drying to obtain the total polysaccharide named as WFVP.

2.2.1. Analytical chromatography on DEAE-cellulose

WFVP (10 mg) was dissolved in distilled water (2 ml). After centrifugation (10,000 rpm, 5 min), the supernatant was loaded on a DEAE-Cellulose (Cl^-) column (1.5 × 14 cm) pre-equilibrated with distilled water. The column was first eluted with distilled water at 1.0 ml/min to yield a single polysaccharide fraction, WFVP-N, and then with a linear gradient from 0.0 to 0.5 M NaCl to obtain WFVP-A (Supplementary Fig. Figure 1A). The eluate was collected at 4 ml per tube and assayed for total sugar and uronic acid contents.

2.2.2. Preparation of WFVP-N by DEAE-cellulose

WFVP (8 g) was dissolved in distilled water (800 ml). After centrifugation (4500 rpm, 15 min), the supernatant was separated by using a DEAE-cellulose column (7.5 × 30 cm, Cl^-). The column was eluted with dH_2O. The eluates were collected, concentrated, and lyophilized to give the neutral fraction WFVP-N (42.3%). WFVP-N was then further purified using gel-permeation chromatography with Sepharose Cl-6B and Sephadex G100 to give homogeneous fraction WFVP-N-b1.

2.2.3. Gel permeation chromatography on Sepharose CL-6B

WFVP-N (100 mg) was dissolved in 0.15 M NaCl (2 ml). The supernatant was loaded onto a Sepharose CL-6B column (1.6 × 100 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 ml/min. The eluate was collected at 3 ml per tube and assayed for total sugar content. The appropriate fractions were combined, dialyzed against distilled water and lyophilized to give WFVP-N-a and WFVP-N-b, respectively (Supplementary Fig. Figure 1B).

2.2.4. Gel permeation chromatography on Sephadex G100

WFVP-N-b (100 mg) was dissolved in 0.15 M NaCl (2 ml), loaded onto a Sephadex G100 (1.6 × 100 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 ml/min. The eluate was collected at 3 ml per tube and assayed for total sugar content. The appropriate fractions were combined, dialyzed against distilled water and lyophilized to give WFVP-N-b1 and WFVP-N-b2 (Supplementary Fig. Figure 1C).

2.3. Analysis of chemical properties

Total carbohydrate content was determined by using the phenol-sulfuric acid method with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). Uronic acid content was determined by using the m-hydroxydiphenyl method with galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Protein content was determined by using the Bradford assay with bovine serum albumin as the standard (Sedmak & Grossberg, 1977). Monosaccharide composition was determined by using high performance liquid chromatography (HPLC) as described by Zhang et al. (2009). Molecular weight distributions were determined by using gel-permeation chromatography on a TSK-gel G-3000P W_{XL} column (7.8 × 300 mm, TOSOH, Japan) coupled to a Shimadzu HPLC system, as described by Zhang et al. (2009). The column was pre-calibrated by using standard dextrans (50 kDa, 25 kDa, 12 kDa, 5 kDa and 1 kDa) using linear regression.

2.4. Methylation analysis

Methylation analysis was carried out according to the method of Needs and Selvendran (1993). In brief, WFVP-N-b1 (10 mg) was dissolved in DMSO (1.5 ml) and methylated with a suspension of NaOH/DMSO (1.5 ml) and iodomethane (2.0 ml). The reaction mixture was extracted with $CHCl_2$, and then the solvent was removed by vacuum evaporation. Completemethylation was confirmed by the disappearance of the −OH band (3200–3400 cm^{-1}) in the FT-IR spectrum. The per-O-methylated polysaccharide was hydrolysed subsequently by using HCOOH (85%, 1 ml) for 4 h at 100 °C and then CF_3COOH (2 M, 1 ml) for 6 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced by using $NaBH_4$ and acetylated (Sweet, Albersheim, & Shapiro, 1975). The resulting alditol acetates were analysed by GC–MS. GC–MS analysis was performed by using Agilent Technologies 7890B GC and 5977B MSD system with an HPe5 capillary column (30 m × 0.32 mm × 0.25 mm). The oven temperature was programed from 120 °C (hold for 1 min) to 210 °C (hold for 2 min) at 3 °C/min, then up to 260 °C (hold for 4 min) at 10 °C/min. Both temperature of inlet and detector were 300 °C. Helium was used as carrier gas. The mass scan range was 50.0–1000.0 m/z.

The degree of branching value (DB) was obtained by using the following equation: DB = NB/(NB + NL), where NB and NL represent the number of branched and linear residues, respectively.